



#9

RECEIVED  
MAY 16 2003  
TECH CENTER 1600/2900

## DECLARATION

I, Masanori Hirota of HIROTA AND ASSOCIATES, residing at Wakabayashi Bldg. 3F, 8-5, Akasaka 2-chome, Minato-ku, Tokyo 107-0052, Japan, do hereby certify that I am conversant with the English and Japanese languages and am a competent translator thereof, and I further certify that to the best of my knowledge and belief the following is a true and correct translation made by me of the document in the Japanese language filed for a patent application in Japan under No. 11/7,365 on January 14, 1999 in the name of JAPAN SCIENCE AND TECHNOLOGY CORPORATION at Tokyo, Japan, entitled: "ENDOTOXIN-UNRESPONSIVE MODEL MOUSE".

Signed this 12 day of May, 2003

Masanori Hirota

#10

PATENT OFFICE  
JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the  
following application as filed with this Office.

Date of Application: January 14, 1999

Application Number: Patent Application No.11-7365

Applicant(s): JAPAN SCIENCE AND TECHNOLOGY  
CORPORATION

Commissioner, Kozo Oikawa (Sealed)  
Patent Office

[Name of Document] Patent Application  
[Case Number] A031P32  
[Filing Date] January 14, 1999  
[Special Notes] Patent Application to which  
the regulation of Patent Law  
30(1) is sought to be applied.  
Patent Application to which  
the regulation of Patent Law  
30(1) is sought to be applied.  
Patent Application to which  
the regulation of Patent Law  
30(1) is sought to be applied.  
[Addressee] Commissioner of Patent Office  
[International Classification] A61K 67/027  
[Inventor]  
[Address or Residence] Room 202, 17-18,  
Onohara-higashi 6-chome,  
Mino-shi, Osaka  
[Name] Shizuo AKIRA  
[Inventor]  
[Address or Residence] Room 301, 4-3,  
Satonaka-cho 2-chome,  
Nishinomiya-shi, Hyogo  
[Name] Kikyoshi TAKEDA  
[Applicant for Patent]  
[Code Number] 396020800  
[Name or Corporation Name] JAPAN SCIENCE AND TECHNOLOGY  
CORPORATION  
[Representative] Moritaka NAKAMURA  
[Agent]

[Code Number] 100107984  
[Patent Attorney]  
[Name or Corporation Name] Masanori HIROTA  
[Indication of Fee]  
[Deposit Account Number] 044347  
[Amount of Payment] 21,000 yen  
[List of Filed Documents]  
[Name of Document] Specification 1  
[Name of Document] Drawing 1  
[Name of Document] Abstract 1  
[Confirmation of Proof] Proof Necessary

[Name of Document] SPECIFICATION

[Title of the Invention] ENDOTOXIN-UNRESPONSIVE MODEL MOUSE

[Scope of Claims]

[Claim 1] An endotoxin-unresponsive non-human animal characterized by that its function of myeloid differentiation primary response gene is deficient on its chromosome.

[Claim 2] The endotoxin-unresponsive non-human animal according to claim 1, wherein the non-human animal is a rodent.

[Claim 3] The endotoxin-unresponsive non-human animal according to claim 2, wherein the rodent is a mouse.

[Claim 4] An assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene; an endotoxin activity of the subject material is assessed.

[Claim 5] An assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene; an interleukin-1 activity of the subject material is assessed.

[Claim 6] An assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene; an interleukin-18 activity of the subject material is assessed.

[Claim 7] An assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene, the non-human animal and a wild-type non-human animal; an endotoxin

activity of the subject material is assessed.

[Claim 8] An assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene, the non-human animal and a wild-type non-human animal; an interleukin-1 activity of the subject material is assessed.

[Claim 9] An assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene, the non-human animal and a wild-type non-human animal; an interleukin-18 activity of the subject material is assessed.

[Claim 10] The assessing method of a subject material according to any one of claims 4 to 9, wherein the non-human animal is a mouse.

#### [Detailed Description of the Invention]

[0001]

#### [Technical Field to Which the Invention Pertains]

The present invention relates to non-human animals being deficient in function of myeloid differentiation primary response (hereinafter "MyD88") gene, and particularly relates to MyD88 knockout mice and assessing methods of an endotoxin activity, an interleukin-1 activity, an interleukin-18 activity and the like in subject materials with the MyD88 knockout mice.

[0002]

#### [Prior Art]

Cytokines are intracellular signal transmitters which play an important role in an immune response. a response upon

infection, hematopoiesis, inhibition of virus infection and tumor cells. Among them, a cytokine which transmits signals between lymphocytes is called interleukin (hereinafter, "IL"). Among ILs, IL-1 is a cytokine which mediates various immune responses and inflammatory responses, and is involved in maintenance of homeostasis of living organisms and produced from various cells such as monocytes, macrophages, keratinocytes, vascular endothelial cells and the like when the living organisms get infected or hurt. It has been known that there are two kinds of IL-1, IL-1 $\alpha$  and IL-1 $\beta$ , both of which combine to the same receptor. It has been also known that IL-1 exerts its function simultaneously with the activation by an antigen to T cell and by mitogens, makes T cells release IL-2, and enhances the expression of IL-2 receptors to induce T cell proliferation, and that it acts on monocytes and macrophages in order to induce the production of TNF, IL-1, IL-6.

[0003]

IL-1 has two kinds of IL-1 receptors (hereinafter "IL-1R"), and both of the IL-1Rs, type I and type II, have three immunoglobulin-like domains in their extracellular domains. Type I receptors express in T cells and connective tissue, and type II receptors express in B cells, myeloids and the like, and it has been known that type I receptors induce NF- $\kappa$ B in nuclei. It has been also known that there is an IL-1 receptor antagonist (hereinafter "IL-1ra") which shows no bioactivity in spite that it binds to IL-1R with the affinity similar to that of IL-1 $\alpha$  and IL-1 $\beta$ , and that it prevents IL-1 from binding to IL-1R competitively.

[0004]

IL-18 is known to promote the production of int rferon-

$\gamma$  (hereinafter "IFN- $\gamma$ "), to enhance the activation of natural kill r cells, to induce the production of IFN- $\gamma$  from T cells in cooperation of IL-12, and to act an important role in a Th1 (IL-2 producting helper T cells) response. Further, it is known that IL-18 has no structural similarity to IL-12 in spite that it has a functional similarity, and has a structural similarity to IL-1. Moreover, it has been also known that IL-18 is produced as an inactive precursor that requires cleavage by IL-1 $\beta$ -converting enzyme (ICE)/caspase1 for its maturation, as in the case of IL-1 $\beta$ , and that IL-18 activates IL-1R-associated kinase (IRAK) and NF- $\kappa$ B.

[0005]

A plurality of molecules showing homology to IL-1R have been identified so far, and signal pathways mediated by IL-1R family is being studied intensively now. It has been known that MyD88 is a cytoplasmic protein comprised of an IL-1R homologous domain and a Death domain, and functions as an adaptor molecule which activates NF- $\kappa$ B by recruiting IRAK to IL-1R complex after IL-1 stimulation, and that MyD88 gene was originally separated as a myeloid differentiation primary response gene, which rapidly induces M1 myeloleukemic cells to macrophages by IL-6-stimulated differentiation.

[0006]

Toxins in bacterial cells being comprised of lipopolysaccharide, which is a major structural component of the outer membrane encompassing peptidoglycan on the surface of Gram-negative bacteria, are called endotoxin, and it has been known that lipopolysaccharide is comprised of lipid called lipid A and various kinds of saccharide which covalently bind to the lipid A. It has been also known that this endotoxin has



a bioactivity mainly involved in fever, decrease of leukocytes and platelet, hemorrhagic necrosis of bone marrow cells, hypoglycemia, induction of IFN, activation of B lymphocyte (immune response cell derived from marrow), and the like.

[0007]

On the other hand, it is known that the function of a specific gene can be analyzed in individual level by using transgenic mice generated by using embryonic stem cells (hereinafter "ES cells"), and knockout mice generated by using gene targeting in which specific genes on genomes are artificially transformed by homologous recombination. In general, gene-deficient mice are called knockout mice, and MyD88 knockout mice have not been known, and moreover, it has not been known that MyD88 knockout mice are unresponsive to endotoxin, either.

[0008]

[An Object to be Attained]

An object of the present invention is to provide MyD88 knockout mice or other non-human animals whose function of MyD88 genes is deficient which can be used for assessing endotoxin activity, IL-1 activity or IL-18 activity of subject materials.

[0009]

[Means to Attain the Object]

The inventors of the present invention have conducted intensive study for attaining the object. They generated MyD88 gene-deficient mice as follows: two exon regions encoding the C-terminal portion of MyD88 gene are replaced with the neomycin-resistant gene by homologous recombination with virus vectors in ES cells and HSV-tk gene was induced into C-terminal side, and ES cell clones doubly resistant of G418 and

gancyclovir were screened; the ES cell clones were microinjected into blastocysts of C57BL/6 mice; MyD88 knockout mice whose function of MyD88 genes is deficient were born through the germline at the expected Mendelian ratios. Then the inventors have found that those MyD88 knockout mice are transgenic mice which grow healthy and show no obvious abnormalities until 20 weeks of age, and have confirmed that those MyD88 knockout mice are unresponsive to endotoxin, and the present invention has thus completed.

[0010]

The present invention relates to endotoxin-unresponsive non-human animals including rodents such as MyD88 knockout mice characterized in that their function of MyD88 genes is deficient. The present invention further relates to an assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal such as MyD88 knockout mice whose function of MyD88 genes is deficient and to a wild-type littermate of the non-human animal; endotoxin activity, IL-1 activity or IL-18 activity of the subject material is assessed.

[0011]

[Mode for Carrying out the Invention]

In the present invention, "deficiency of MyD88 gene function" means that a part of or a whole of MyD88 gene on a chromosome is deficient and the function to express MyD88, which is expressed in wild-types, is lost. Examples of a non-human animal whose function of MyD88 gene is deficient include a rodent such as a rat or the like whose function of MyD88 gene is deficient other than MyD88 knockout mice. As examples of the non-human animals whose function of MyD88 gene is deficient,

the ones gen rated at the expected Mendelian ratio are preferably exemplified considering that wild-type littermates of MyD88-deficient type are obtainable and that precise comparative experiments can be conducted with the animals. With an example of MyD88 knockout mice, a generating method of the non-human animal whose function of MyD88 gene is deficient will now be explained.

[0012]

MyD88 gene can be screened by using gene fractions obtained from a mouse genomic library through PCR. The screened MyD88 gene is subcloned with virus vector or the like, and can be characterized by restriction enzyme mapping and DNA sequencing. Then, a targeting vector is replaced with a marker gene such as a neomycin resistance gene or the like, and subsequently the targeting vector is linearized and transfected with embryonic stem cells (ES cells), and then clones, for instance, clones which show resistance to G418 are screened, and obtained targeting ES clones are microinjected into blastocysts of mice. Chimeric mice are mated with female mice, and thus obtained heterozygous mice are intercrossed in order to obtain homozygous mice, then the object MyD88 knockout mice and wild-type mice are generated at the expected Mendelian ratio.

[0013]

It is possible to confirm that the obtained MyD88 knockout mice are unresponsive to endotoxin, for example, by injecting LPS, which is endotoxin, into MyD88 knockout mice by intravenous injection or the like, and then measuring bioactivity of endotoxin such as fever, shock, decrease of leukocytes or platelet, hemorrhagic necrosis of bone marrow cells,

hypoglycemia, induction of IFN, activation of B lymphocyte (immune response cell derived from marrow) or the like. The MyD88 knockout mice of the present invention show lower responsiveness to endotoxin than C3H/HeJ mice, which have been known as being hyporesponsive to endotoxin so far, and no shock symptom has been observed. As LPS-unresponsive mice, the MyD88 knockout mice can be used as useful model for elucidating action mechanisms of endotoxin, and for establishing a treatment method for endotoxin shock.

[0014]

The endotoxin activity of the subject material can be assessed precisely by using the MyD88 knockout mice or the MyD88 knockout mice and wild-type mice, preferably wild-type littermate, as control. By precisely assessing endotoxin activity of a subject material, it becomes possible to obtain useful information for developing antagonists to endotoxin or other such pharmaceuticals which can suppress the shock or fever caused by endotoxin.

[0015]

IL-1 activity of a subject material can be assessed after administering the subject material to the MyD88 knockout mice of the present invention or the MyD88 knockout mice and wild-type mice, preferably wild-type littermate of the MyD88 knockout mice. Examples of IL-1 activity as an object of assessment include mitogens such as phytohemagglutinin (PHA), concanavalin A (Con A) and the like, proliferation inducing activity of T cells caused by co-stimulation with IL-2 at a low concentration, and activity which induces the production of TNF, IL-1 and IL-6 by working on monocytes and macrophages.

[0016]

By using the MyD88 knockout mice or the MyD88 knockout mice and wild-type mice, preferably wild-type littermate, as control, the IL-1 activity of the subject material can be assessed precisely, and the relationship between IL-1 and the illness in disease model mice can be examined. It becomes possible to obtain useful information for developing pharmaceuticals which can cure diseases such as rheumatoid arthritis caused by overexpression of IL-1, a graft-versus-host disease, asthma and the like by precisely assessing IL-1 activity of a subject material and by analyzing the involvement of IL-1 in disease model mice.

[0017]

IL-18 activity of a subject material can be assessed after administering the subject material to the MyD88 knockout mice of the present invention or the MyD88 knockout mice and wild-type mice, preferably wild-type littermate of the MyD88 knockout mice. Examples of IL-18 activity as an object of assessment include activity which promotes production of IFN- $\gamma$ , activity which enhances activity of NK cells, activity which induces production of IFN- $\gamma$  from T cells in cooperation with IL-12, and action which activates IRAK or NF- $\kappa$ B.

[0018]

By using the MyD88 knockout mice or the MyD88 knockout mice and wild-type mice, preferably wild-type littermate, as control, the IL-18 activity of the subject material can be assessed precisely. It becomes possible to obtain useful information for developing pharmaceuticals which can cure diseases caused by overproduction of IL-18, such as I type diabetes, a graft-versus-host disease and the like by precisely assessing IL-18 activity of a subject material.

[0019]

The present invention will be explained more specifically with examples below, but the technological scope of the present invention is not limited to these examples.

Example 1 (Generation of MyD88 knockout mice)

A MyD88 gene was screened from a 129/SvJ mouse genomic library (Stratagene), subcloned into pBluescript vector (Stratagene), and characterized by restriction enzyme mapping and DNA sequencing. A targeting vector was constructed by replacing the 1.0 kb genomic fragment with a neomycin resistance gene from pMC1-neo (Stratagene). The replaced genomic fragment contained 2 exons encoding the domain that resembles the cytoplasmic domain of the IL-1RacP (receptor accessory protein). The neomycin resistance gene was flanked by the 1.1 kb 5' genomic fragment and the 5.2 kb 3' fragment. Then, an HSV-tk cassette was introduced into the 3' end of the genomic fragment. E14.1 ES cells were transfected with the linearized targeting vector and selected with G418 and gancyclovir. Doubly resistant 176 clones were screened for homologous recombination by PCR and 33 clones were verified by Southern blot analysis using the probe indicated in Fig. 1.

[0020]

Three independently identified targeted ES clones were microinjected into the blastocysts of C57BL/6 mice. Thus obtained chimeric mice were mated with C57BL/6 female mice to produce heterozygous mice. The Heterozygous mice were intercrossed to obtain homozygotes, and MyD88-deficient were born at the expected Mendelian ratios (+/+ : +/- : -/- = 52:93:53) from the intercross. The MyD88 knockout mice of the present invention grew healthy and showed no obvious abnormalities

until 20 weeks of age. Northern blot analysis was performed to confirm that the inactivation of the MyD88 gene was caused by mutation. MyD88 mRNA could not be detected in the liver and the spleen of the MyD88-deficient mice. Flow cytometric analysis of CD3, B220, CD4, and CD8 in thymus, spleen, and lymph node showed that lymphocyte composition was not altered in the MyD88 knockout mice in comparison with wild-type mice.

[0021]

Example 2 (Unresponsiveness of MyD88 knockout mice to Endotoxin)

1mg of LPS derived from *Escherichia coli* (O55:B5) was administered to 10 MyD88 knockout mice of the present invention, and endotoxin-unresponsiveness was examined through the survival ratio of the mice. 10 wild-type littermates were used as control. The results are shown in Fig. 2. It is confirmed by Fig. 2 that though the wild-type mice have responded to LPS and all of them died within 4 days after administration, none of the MyD88 knockout mice of the present invention have died within 4 days after LPS administration, and that the mice are endotoxin-unresponsive.

[0022]

Example 3 (Impaired IL-1-mediated functions in MyD88 knockout mice)

$1 \times 10^5$  of thymocytes of the MyD88 knockout mice of the present invention were cultured in 96-well plates for 72 hours with mixtures containing 2  $\mu$ g/ml of phytohemagglutinin (PHA), which is a costimulant of IL-1 for T cell proliferation, 2.5  $\mu$ g/ml of concanavalin A (ConA), 2 ng/ml of IL-2 respectively, and 100 U/ml of IL-1 $\beta$  (Genzyme), and T cells were proliferated. Proliferation of T cells were examined by measuring [ $^3$ H] amount

of [<sup>3</sup>H] thymidine taken into the cells. As a result, thymocytes of wild-type littermates displayed enhanced proliferation when cultured with PHA, ConA or IL-2 in the presence of IL- $\beta$ , however, thymocytes of the MyD88 knockout mice of the present invention show almost no enhanced proliferation (see Fig. 3). It has been found that similar results could be obtained even when splenocytes were used instead of thymocytes.

[0023]

Further, thymocytes of MyD88 knockout mice of the present invention were cultured with 10 ng/ml of phorbol 12-myristate 13-acetate paramethoxyamphetamine (PMA) or 2.5  $\mu$ g/ml of Con A in the presence of 20 ng/ml of IL-2 (Genzyme) in a same manner as above-mentioned, and enhancement of proliferation was examined. There was no difference between thymocytes of MyD88 knockout mice of the present invention and of wild-type littermates in their proliferation as to the reaction of IL-2 and PMA or Con A (see Fig. 3). These results indicate that IL-1-mediated growth signal of T cells was impaired in the thymocytes of MyD88 knockout mice of the present invention.

[0024]

MyD88 knockout mice of the present invention were intravenously injected with 1  $\mu$ g of IL- $\beta$  (Genzyme), and 2 hours later liver and sera were taken. Total RNA was extracted from the liver using Trizol reagent (GIBCO). This RNA (10 $\mu$ g) was electrophoresed and transferred to a nylon membrane, then Northern blot analysis was conducted with <sup>32</sup>P-labelled cDNA for acute phase proteins such as serum amyloid A (SAA-I), serum amyloid P (SAP), and haptoglobin (HP). In comparing IL-1-induced increase of mRNA expression in wild-type littermates and in MyD88 knockout mice of the present invention, increase



of induction was observed in wild-type mice, but not observed in MyD88 knockout mice.

[0025]

Because IL-1 induces production of acute phase proteins such as tumor necrosis factor (TNF) or IL-6, and proinflammatory cytokines, increase of TNF and IL-6 concentrations in serum taken from MyD88 knockout mice of the present invention and wild-type littermates by the above-stated method were measured by ELISA. As a result, TNF and IL-6 concentrations increased by IL-1 $\beta$  in wild-type mice, while neither TNF nor IL-6 concentration increased by IL-1 $\beta$  in MyD88 knockout mice (see Fig.4).

Thus, IL-1-mediated major biological functions has been found to be severely impaired in MyD88 knockout mice of the present invention.

[0026]

Example 4 (Impaired IL-18-mediated functions in MyD88 knockout mice)

It has been well known that IL-18 enhances lytic activity of NK cells. Splenocytes from MyD88 knockout mice of the present invention and wild-type littermates were cultured in the presence or absence of 20 ng/ml of IL-18 (Hayashibara Biochemical Laboratories, Inc.) for 24 hours with <sup>51</sup>Cr-labelled yeast artificial chromosome (hereinafter "YAC-1") targeting cells. 4 hours later, released <sup>51</sup>Cr in supernatants were counted by a gamma counter. As a result, when splenocytes were cultured in the presence of IL-18 in vitro, lytic activity to YAC-1 targeting cells was dramatically enhanced in wild-type mice, but it was not enhanced in MyD88 knockout mice. When IL-2 was used instead of IL-18, lytic activity was also enhanced in

splenocytes of MyD88 knockout mice of the present invention (see Fig.5).

[0027]

Further, splenocytes of MyD88 knockout mice of the present invention and their wild-type littermates were stimulated by 20 ng/ml of IL-18 and cultured for 24 hours in vitro, then production of IFN- $\gamma$  in culture supernatants was measured by ELISA. As a result, production of IFN- $\gamma$  was induced in wild-type mice, however, production of IFN- $\gamma$  was not observed in MyD88 knockout mice of the present invention (see Fig.5).

[0028]

Splenic T cells of MyD88 knockout mice of the present invention and their wild-type littermates, which were purified to 95% or over, were cultured on anti-CD3 antibody (20  $\mu$ g/ml)(PharMingen)-coated plates in the presence of 2 ng/ml IL-12. 4 days after the onset of culture, cells were harvested and washed with Hanks' balanced salt solution. The washed cells ( $2 \times 10^5$ ) were stimulated and cultured again on anti-CD3 antibody (20  $\mu$ g/ml)-coated 96-well plates for 24 hours with 20 ng/ml of IL-18 or 2 ng/ml of IL-12. Concentration of IFN- $\gamma$  in culture supernatants was determined by ELISA and compared. The result indicates that Splenic T cells of MyD88 knockout mice of the present invention cannot enhance IL-18-responsive production of IFN- $\gamma$  (see Fig.6).

[0029]

MyD88 knockout mice of the present invention and their wild-type littermates were intraperitoneally injected with 500  $\mu$ g of heat-killed *Propionibacterium acnes* (*P. acnes*). Seven days after injection, T cells were purified from spleen, then

cultured and stimulated on anti-CD3 antibody (20 µg/ml)-coated 96-well plates for 24 hours in the presence or the absence of 20 ng/ml of IL-18. Concentration of IFN- $\gamma$  in culture supernatants was determined by ELISA. MyD88 knockout mice of the present invention and their wild-type littermates were intravenously injected with 2 mg of Bacillus Calmette-Guérin (BCG) (Kyowa). 14 days after injection, T cells were purified from spleen, then cultured and stimulated for 24 hours, as described above, subsequently concentration of IFN- $\gamma$  was measured. As a result, in both cases, high level of IFN- $\gamma$  production in response to IL-18 was observed in wild-type mice, but production level of IFN- $\gamma$  could not be enhanced in the presence of IL-18 in MyD88 knockout mice of the present invention (see Fig.6).

[0030]

These results demonstrate that MyD88 knockout mice of the present invention are defective in Th1 cell development in vivo as is the case with IL-18-deficient mice, and that their major biological activities mediated by IL-18 were completely abolished.

[0031]

Next, it was examined whether the dominant negative MyD88 mutant blocked IL-18-induced NF- $\kappa$ B activation as well. COS-7 cells were transiently transfected with MyD88 (amino acid 152-296) expression vector together with NF- $\kappa$ B-dependent luciferase reporter gene, and luciferase activity after IL-18 treatment was measured. Coexpression of MyD88 blocked IL-18-induced activation almost completely (see Fig. 7).

[0032]

Because IL-18 activates AP-1-dependent gene information,

whether MyD88 (amino acid 152-296) also acted as a dominant negative mutant of IL-18-induced AP-1 activation was investigated. Stimulation with IL-18 induced an approximately 3- to 4-fold increase in AP-1 activity, and this activation was blocked by coexpression of MyD88 (amino acid 152-296) (see Fig. 7). These results show that MyD88 is involved in IL-18-induced activation of both NF- $\kappa$ B and AP-1.

[0033]

Further, whether IL-18-induced activation of NF- $\kappa$ B was observed in MyD88-deficient cells was examined. Splenic T cells cultured in the presence of IL-12 and anti-CD3 antibody for 4 days were starved for 3 hours and then stimulated with IL-18. Nuclei extracted from the stimulated cells were analyzed by a gel mobility shift assay using a specific probe containing NF- $\kappa$ B binding site. IL-18-induced NF- $\kappa$ B DNA binding activity was detected in the nuclear extracts from wild-type cells but not from MyD88-deficient cells. On the other hand, treatment of wild-type or MyD88-deficient thymocytes with TNF $\alpha$  resulted in almost the same levels of NF- $\kappa$ B DNA binding activity, demonstrating that the impaired IL-18-induced NF- $\kappa$ B activity in MyD88-deficient cells was not due to the abnormal function or impairment of regulating ability of NF- $\kappa$ B.

[0038]

In addition to induction of NF- $\kappa$ B activation, IL-1 is also known to activate c-Jun N-terminal kinase (JNK). To test whether IL-18 induces JNK activation, an in vitro kinase assay was carried out using GST-c-Jun-fusion protein as a substitute. Treatment with IL-18 induced JNK activation in Th1-developing cells of wild-type mice. However, IL-18-induced JNK

activation was not observed in MyD88-deficient cells. By contrast, normal activation of JNK was observed in MyD88-deficient cells treated with TNF- $\alpha$ . IL-18-induced NF- $\kappa$ B and JNK activation was impaired in MyD88-deficient mice. These results demonstrate that MyD88 is essential for IL-18-induced activation of both NF- $\kappa$ B and JNK.

[0035]

[Effect of the Invention]

The MyD88 knockout mouse of the present invention is unresponsive to endotoxin and its biological function mediated by IL-1 and IL-18 is deficient. Therefore, by using the MyD88 knockout mouse of the present invention, it becomes possible to assess endotoxin activity, IL-1 activity and IL-18 activity in subject materials, and to obtain useful information for development of medicines for diseases caused by excessive production of endotoxin, IL-1, IL-18 or receptors of these materials.

[Brief Description of Drawings]

[Fig. 1]

This is a graph showing gene maps of the MyD88 knockout mice and the wild-type mice of the present invention.

[Fig. 2]

This is a graph showing survival indices of the MyD88 knockout mice and the wild-type mice of the present invention having an injection of LPS derived from *Escherichia coli*.

[Fig. 3]

This is a graph showing the results of T cell proliferation mediated by IL-1 in the MyD88 knockout mice and the wild-type mice of the present invention.

[Fig. 4]

This is a graph showing the results of IL-1-induced TNF and IL-6 levels in blood in the MyD88 knockout mice and the wild-type mice of the present invention.

[Fig. 5]

This is a graph showing the results of NK cell activation mediated by IL-18 in the MyD88 knockout mice and the wild-type mice of the present invention.

[Fig. 6]

This is a graph showing the results of the production of IFN- $\gamma$  stimulated by IL-12 and IL-18 in the MyD88 knockout mice and the wild-type mice of the present invention.

[Fig. 7]

This is a graph showing that the mutation of dominant negative MyD88 is involved in IL-18-induced NF- $\kappa$ B activity and AP-1 activity.

[Name of Document] Abstract

[Abstract]

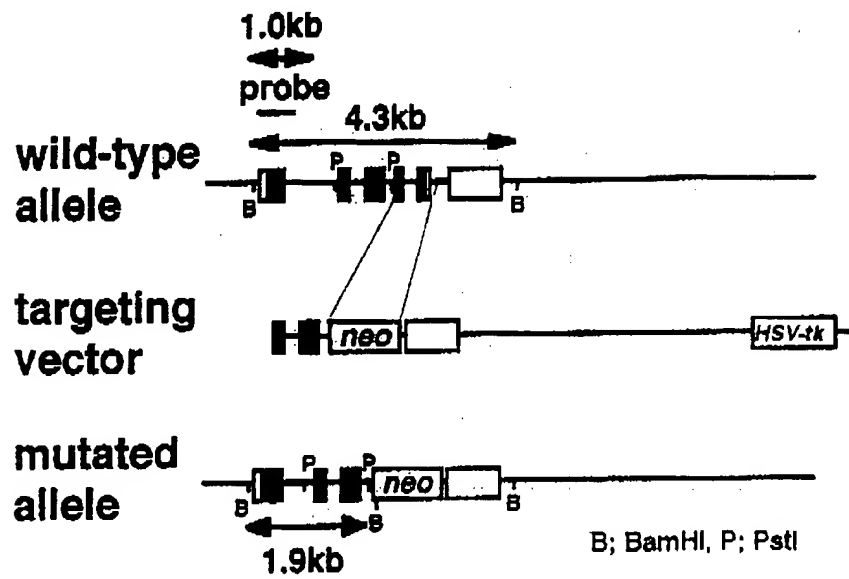
[The Object] The object of the present invention is to provide endotoxin-unresponsive model mice which is deficient in function of myeloid differentiation primary response (MyD88) genes and which can be used for assessing endotoxin activity, interleukin-1 activity or interleukin-18 activity of subject materials.

[Solving Means] Generating MyD88 knockout mice which can be used for assessing endotoxin activity, interleukin-1 activity or interleukin-18 activity of the subject material after administering the subject material to MyD88 knockout mice whose function of MyD88 genes is deficient and to wild-type littermates of them.

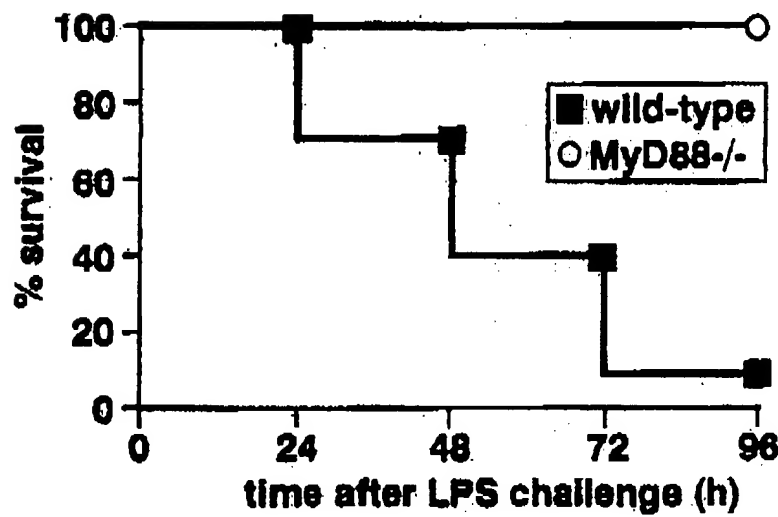
[Selected Drawing] Fig. 1

[Nam of Document] Drawing

[Fig. 1]

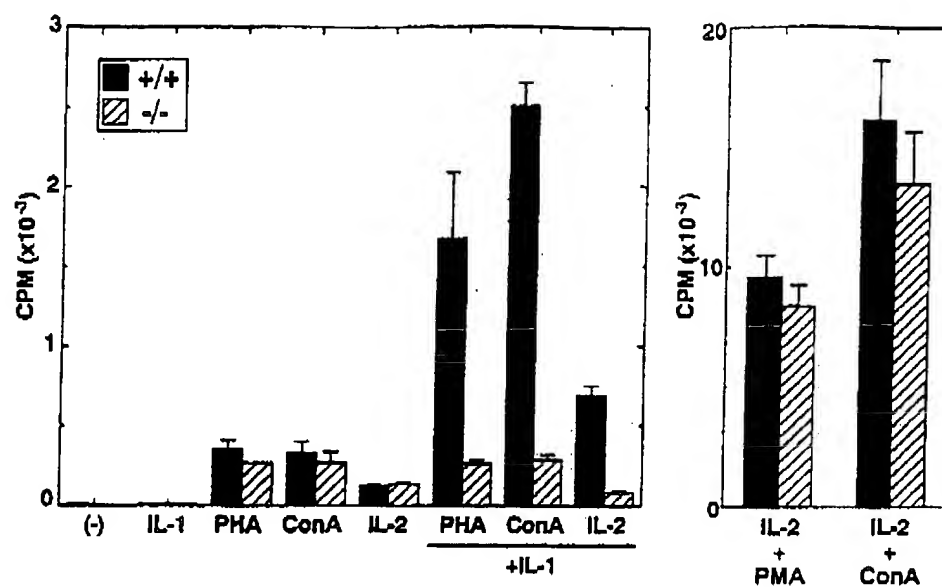


[Fig. 2]

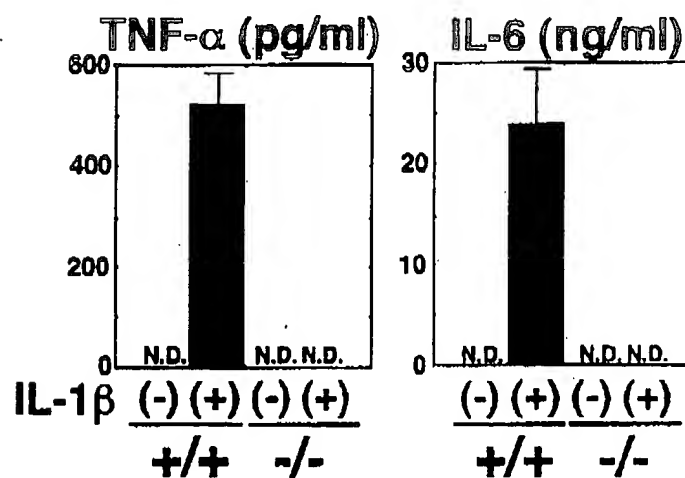




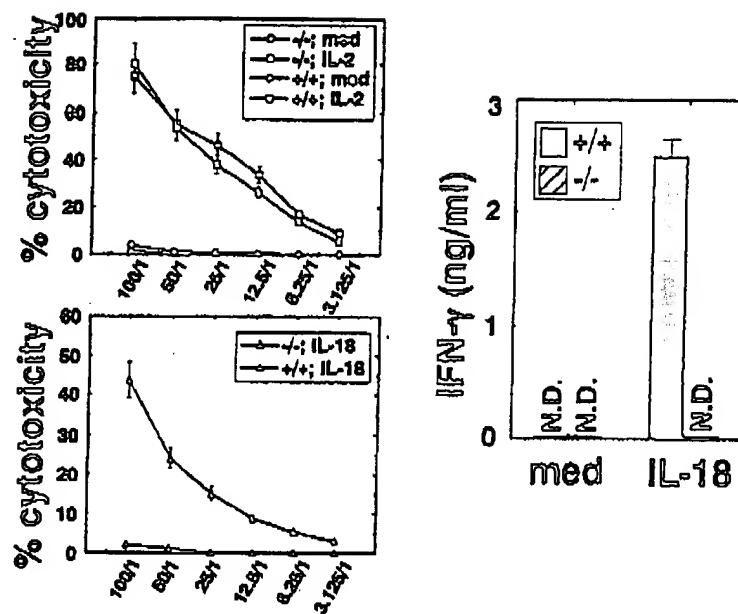
[Fig. 3]



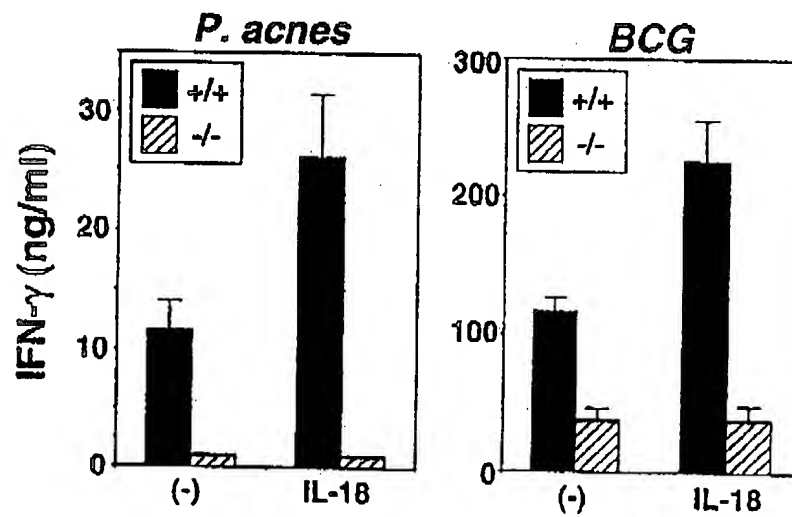
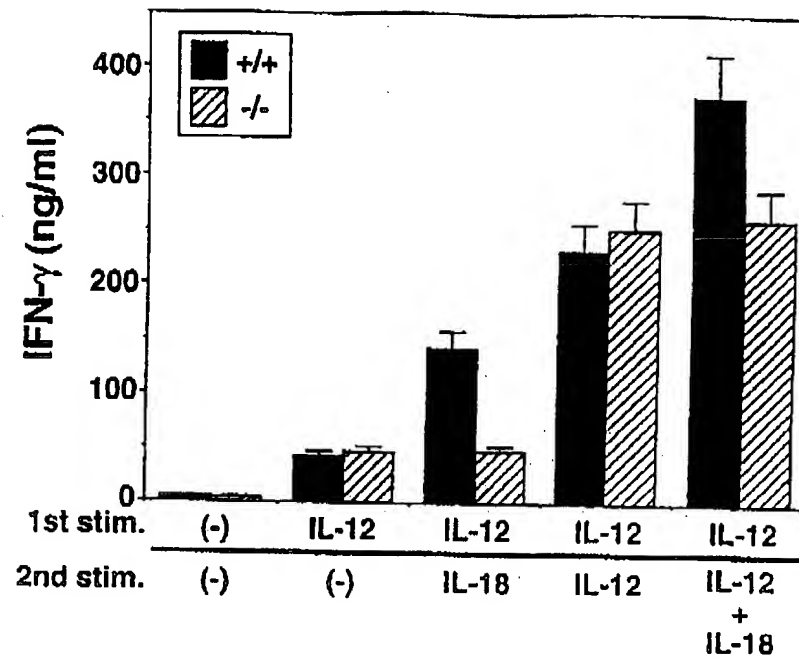
[Fig. 4]



[Fig. 5]



[Fig. 6]



[Fig. 7]

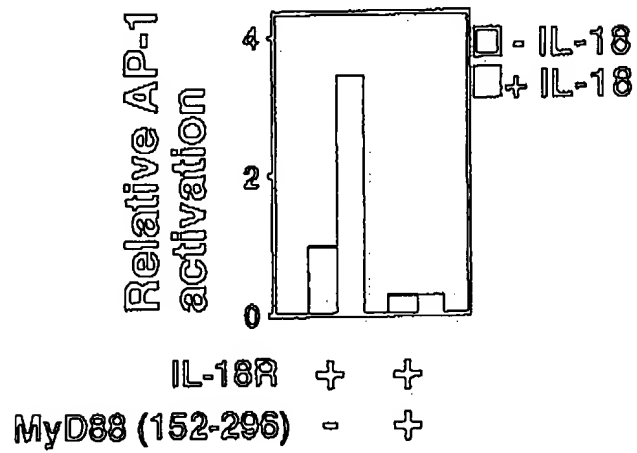
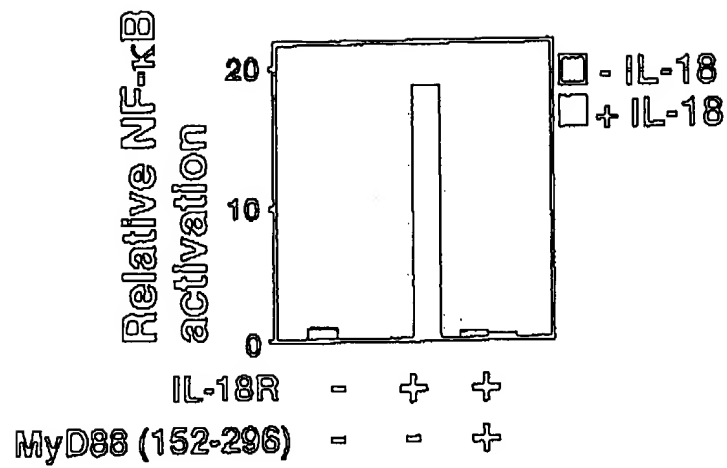


FIG. 19

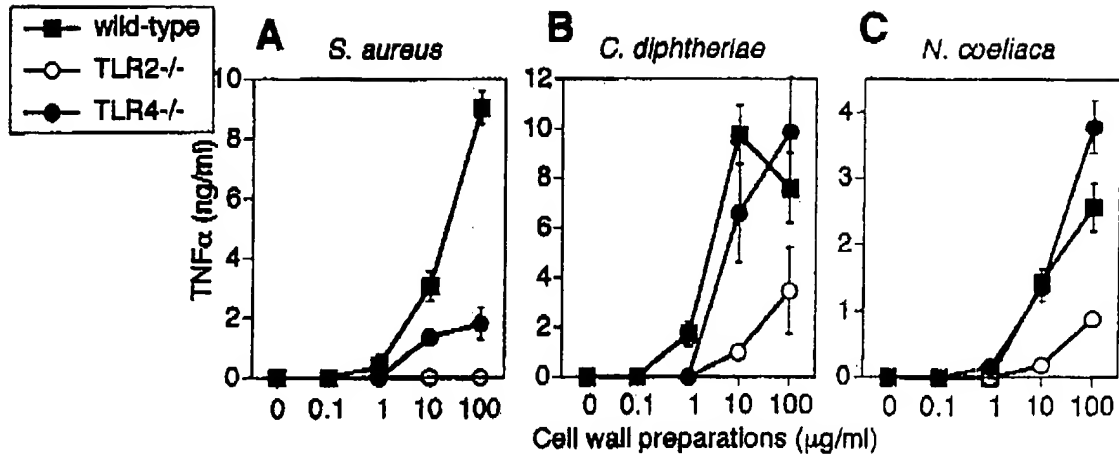


FIG. 20

